

Seven-up acts as a temporal factor during two different stages of neuroblast 5-6 development

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SUMMARY

Drosophila embryonic neuroblasts generate different cell types at different time points. This is controlled by a temporal gene cascade of Hb->Kr->Pdm->Cas->Grh, which acts to sequentially dictate distinct competence windows. In addition, Seven-up (Svp), a member of the nuclear hormone receptor family, acts early in the temporal cascade, to ensure the transition from Hb to Kr, and has been referred to as a “switching factor”. However, Svp is also expressed in a second wave within the developing CNS, but here, the possible role of Svp has not been previously addressed. In a genetic screen for mutants affecting the last-born cell in the embryonic NB5-6T lineage, the Ap4/FMRFamide neuron, we have isolated a novel allele of *svp*. Expression analysis shows that Svp is expressed in two distinct pulses in NB5-6T, and mutant analysis reveals that *svp* plays two distinct roles. In the first pulse, *svp* acts to ensure proper down-regulation of Hb. In the second pulse, which occurs in a Cas/Grh double-positive window, *svp* acts to ensure proper sub-division of this window. These studies show that a temporal factor may play dual roles, acting at two different stages during the development of one neural lineage.

INTRODUCTION

Neural progenitor cells, in both vertebrates and invertebrates, go through temporal competence changes, evident by the generation of different classes of neurons and glia at different time points (Okano and Temple, 2009). These programmed changes are likely to be controlled by a combination of both extrinsic and intrinsic cues, and evidence points to the existence of both mechanisms in vertebrates and invertebrates. With respect to intrinsic cues, major progress has been made in the *Drosophila melanogaster* (*Drosophila*) system, in particular in the embryonic ventral nerve cord (VNC). Here, temporal competence changes have been shown to be under control of an intrinsic temporal cascade of transcription factors, the temporal gene cascade (Brody and Odenwald, 2002; Jacob et al., 2008; Pearson and Doe, 2004). This cascade consists of the sequential expression, and function, of the Hunchback (Hb), Kruppel (Kr), Nubbin and Pdm2 (denoted collectively Pdm herein), Castor (Cas) and Grainyhead (Grh) transcription factors, in a Hb->Kr->Pdm->Cas->Grh cascade. The precise progression of this cascade is an effect of mutually activating and repressing actions of the factors upon each other. In addition, studies have also identified factors that facilitate this progression i.e., “switching factors”. Here, the *seven-up* (*svp*) and *distal antenna/distal antenna related* (collectively referred to as *dan* herein) genes have been shown to play important roles ensuring the switch from Hb->Kr, by suppressing Hb (Kanai et al., 2005; Kohwi et al., 2011; Mettler et al., 2006). Both Svp and Dan display a second wave of expression, but their function here is unknown. Finally, our previous studies have also identified the existence of so-called “sub-temporal” genes, which act downstream of the temporal genes, do not regulate temporal genes, and act to sub-divide larger temporal windows (Baumgardt et al., 2009). However, in spite of the progress in understanding temporal competence changes, it is not clear how neuroblasts switch from one competence window to the next, how window size is controlled and how windows are sub-divided. Moreover, recent mathematical modeling of the temporal cascades, indicate the existence of additional players involved in the temporal competence changes observed in vivo (Nakajima et al., 2010).

To address these issues, we are using the *Drosophila* embryonic thoracic neuroblast 5-6 (NB5-6T) as a model. This neuroblast, which can be readily identified by the specific expression of reporter genes under the control of an enhancer fragment from the ladybird early gene (*lbe(K)*) (De Graeve et al., 2004), is generated in each of the six thoracic VNC hemisegments. Each NB5-6T produces a mixed lineage of 20 cells, and the four last cells to be born are a set of four

interneurons expressing the Apterous (Ap) LIM-homeodomain transcription factor; the Ap neurons (Baumgardt et al., 2009). The four Ap neurons can be further sub-divided into three different neuronal sub-types; the Ap1/Nplp1 and Ap4/FMRFa neurons, expressing the Nplp1 and FMRFamide neuropeptides, respectively, and the Ap2/Ap3 interneurons (Fig. 1A-B) (Baumgardt et al., 2009; Baumgardt et al., 2007; Benveniste et al., 1998; Park et al., 2004). The birth order of each Ap neuron is stereotyped, and their number refers to their birth-order. The unique expression of different *lbe(K)* reporters, allowing for the selective identification and analysis of this one lineage, combined with the selective expression of Ap, Nplp1 and FMRFa, and several other markers, makes this lineage particularly useful for addressing temporal competence changes with single-lineage and single-cell resolution.

Previously, we resolved the lineage progression of NB5-6T, and determined the expression and function of the temporal and sub-temporal genes within this lineage, with particular emphasis upon Ap neuron generation (Baumgardt et al., 2009). These studies revealed that Ap neurons are generated in a Cas/Grh late temporal window, and that both genes are critical for specification of Ap neurons (Fig 1B). Grh plays a highly specific role and is critical for specification of the last-born neuron, Ap4/FMRFa. Cas plays a more central role, and activates a number of downstream targets including genes that determine Ap neuron fate, such as *collier* (*col*; Flybase *knot*), and genes that act to sub-divide the Ap window. The latter genes, *squeeze* (*sqz*) and *nab*, were denoted sub-temporal genes, and act to down-regulate Col in the later born Ap neurons. This allows Col to play its two distinct roles in Ap neurons; first specifying a generic Ap neuron fate, and second to specifically dictate final Ap1/Nplp1 fate. Thus, down-regulation of Col in the Ap2, Ap3 and Ap4/FMRFa neurons is critical for allowing these alternate Ap neuron fates to be established at later stages. However, although elaborate in their nature, these temporal and sub-temporal cascades fall short of explaining the precision in Ap neuron sub-type specification – the highly stereotyped generation of exactly one Ap1/Nplp1 neuron, followed by two Ap2/3 neurons, and finally the Ap4/FMRFa neuron i.e., a precise 1-2-1 sub-type specification, at the end of the NB5-6T lineage.

To address this issue, we have conducted a large-scale forward genetic screen, using an *FMRFa-EGFP* reporter transgene. One of the mutants identified in this screen, by its loss of *FMRFa-EGFP* expression, was mapped to *svp*. Our analysis of Svp expression demonstrates that it is expressed in two distinct pulses in the NB5-6T lineage. In the early pulse, *svp* plays its

previously identified role i.e. acting as a switching factor by suppressing Hb, thereby allowing for the progression of the temporal cascade during the early parts of the lineage. However, in the latter part of the lineage, as *Svp* is re-expressed in a second wave throughout the VNC, *Svp* is expressed specifically in the Ap neurons. Its second pulse of expression is dynamic, initially commencing in all four Ap neurons but rapidly being down-regulated in the first- and last-born Ap neurons, Ap1/Nplp1 and Ap4/FMRFa, respectively. Mutant analysis shows that in the second pulse, *svp* acts as a sub-temporal gene, by down-regulating Col, thereby allowing for the establishment of alternate Ap cell fates. The second function of *svp* in this lineage – acting as a sub-temporal gene – is similar to the role of *sqz* and *nab*. However, in contrast to *sqz* and *nab*, misexpression of *svp* results not only in ectopic down-regulation of Col in the Ap1/Nplp1 neuron, and suppression of this fate, but also in suppression of the last-born neuron fate, Ap4/FMRFa. Thus, *svp* acts to “gate” the central Ap window (Ap2/3) by suppressing the two cell fates temporally adjacent to this i.e., the Ap1/Nplp1 and Ap4/FMRFa fates. Hence, *svp* adds insight to the complex regulatory cascades involved in the precise 1:2:1 temporal generation of the three distinct Ap neuron sub-types at the end of NB5-6T lineage.

These studies show that one gene can have dual temporal function in one neural lineage, acting first as a switching factor by regulating canonical temporal genes, and secondly by acting as a sub-temporal gene, “micro-managing” the Ap window.

RESULTS

A screen for genes controlling the specification of the Ap4/FMRFamide neuron, the last-born neuron in the NB 5-6T lineage, identifies *seven-up*

Previous studies have identified a number of regulatory genes and pathways acting, between Stage 12 (St12) and 18 hours after egg-laying (18hAEL), to specify the Ap neurons (Fig 1A-B) (Allan et al., 2005; Allan et al., 2003; Baumgardt et al., 2009; Baumgardt et al., 2007; Benveniste et al., 1998; Hewes et al., 2003; Miguel-Aliaga et al., 2004; Park et al., 2004). To further understand the development of this lineage and the specification of the Ap neurons, we have conducted a forward genetic screen, looking for genes affecting a *FMRFa-EGFP* reporter (C.U. and S.T. to be described elsewhere). One mutant identified in this screen, *11C115*, was mapped by deficiency mapping to the *seven-up* (*svp*) gene (Fig. 1C-D). Several *svp* allelic combinations all displayed a near complete loss of FMRFa expression (see below). For this study we have used *svp*^{*l*}, which is a strong (amorph) EMS allele (Hiromi et al., 1993; Kerber et al., 1998), and our novel *svp*^{*11C115*} allele, which also acts as a strong allele.

Seven-up is expressed in two pulses in the NB5-6T lineage

Previous studies revealed that Svp is expressed in two pulses during VNC development (Kanai et al., 2005; Kohwi et al., 2011). We also noted a distinct biphasic appearance of Svp expression in the VNC (not shown). In line with previous studies, we found that *svp* mutants displayed an early failure to down-regulate Hb in general in the VNC (Fig. 2A-B). In the NB5-6T specifically, we also observed two distinct windows of Svp expression (Fig. 3). The early pulse of Svp expression commences at St10, and lasts until St11 (Fig. 3A-C). This brief pulse of Svp expression correlates well with a role for *svp* in down-regulating Hb, and indeed, we found that *svp* mutants often displayed a failure to down-regulate Hb also in the NB5-6T (Fig. 2C-D). However, the failure to down-regulate Hb was only partially penetrant, and 64% of hemisegments did not show persistent Hb expression (Fig. 2E).

The second pulse of Svp expression commenced at St14 in the neuroblast, and was dynamic during subsequent stages (Fig. 3G-M). At late St14, Svp expression was observed in the newly-born Ap4 cell and at St15 in all four Ap neurons. At St16, expression was lost from Ap1, and at St17 from Ap4, while Ap2 and Ap3 maintained Svp expression until end of

embryogenesis (Fig. 3G-M). Thus the second pulse of Svp expression fits well with a role in controlling Ap neuron specification.

***seven-up* mutants display defects in Apterous neuron differentiation**

Mutations in *svp* resulted in a complete loss of *FMRFa-EGFP* expression (Fig. 1D). To further validate this finding, we analyzed expression of the propeptide for the FMRFa neuropeptide itself, and observed a complete loss also of FMRFa in the lateral thoracic areas (Fig 4B, 4F). In contrast, the anterior SE2 FMRFa neurons show an increase in cell numbers (Fig. 4B, 4F) (Losada-Perez et al., 2010). To address whether or not Ap neurons were generated in *svp* mutants, we utilized the Eyes absent (*Eya*) marker, a selective marker for Ap neurons at late embryonic stages (Miguel-Aliaga et al., 2004). In *svp* mutants, we found two very different outcomes. In one subset of hemisegments, we find a complete loss of *Eya* expression (29%; n=102 hemisegments)(Fig. 4A, 4E). In contrast, in another subset of hemisegments, we found that Ap neurons are indeed generated in *svp* mutants, and in fact we observed a prevalent increase in Ap neuron numbers, from 4 to ~6 cells (71%; n=102 hemisegments; Fig. 4E and 4P). These numbers correspond to the number of mutant hemisegments where down-regulation of Hb has not occurred properly (Fig. 2D-E).

Focusing on the hemisegments where Ap neurons could be identified by their *Eya* expression, we analyzed expression of FMRFa and Nplp1, and found that they were strongly affected also in *Eya*-expressing hemisegments, but in opposite ways: FMRFa was typically absent while Nplp1 was often ectopically expressed (Fig. 4I-J, 4L-M, 4P). Next, we analyzed expression of the Dimmed (*Dimm*) basic-helix-loop-helix transcription factor, a key regulator of the general neuropeptide cell identity which is normally expressed in both the Ap1/Nplp1 and Ap4/FMRFa neuropeptide neurons (Allan et al., 2005; Baumgardt et al., 2007; Hewes et al., 2003; Park et al., 2004). In *Eya*-expressing hemisegments, we found that *svp* mutants displayed ectopic *Dimm* expression (Fig. 4K, 4N, 4P).

We find that *svp* mutants display two separate phenotypes in the NB5-6T lineage. At early stages, the down-regulation of the first temporal factor, Hb, often fails. At later stages, this failure of proper temporal progression leads to a failure to specify Ap neurons, as evident by the loss of *Eya* expression in one third of hemisegments. However, in the other two thirds of hemisegments, Ap neurons are indeed generated. But there are three apparent phenotypes at

these later stages: we often observe 1-2 extra Ap neurons, there are extra Ap1/Nplp1 neurons, and a loss of the Ap4/FMRFa cell fate (Fig. 4O).

***seven-up* positively and negatively controls Apterous neuron determinants**

To address the role of *svp* in the Ap window in more detail, we analyzed expression of a number of other genes critical for proper Ap neuron specification. These included the temporal genes *cas* and *grh*, and the sub-temporal genes *sqz* and *nab* (Baumgardt et al., 2009; Terriente Felix et al., 2007). We observed a weak effect upon Cas expression, with a small numerical loss (Fig. 5A, 5G, 5M). Grh expression was somewhat weaker but was numerically unaffected (Fig. 5B, 5H, 5M). Thus both of these temporal genes are still expressed in *svp* mutants. Of the sub-temporal factors, Sqz was largely unaffected although it is expressed in an occasional extra cell (Fig. 5C, 5I, 5M). In contrast, Nab was completely lost from Ap neurons in *svp* (Fig. 5D, 5J, 5M).

Next, we analyzed the expression of the Ap neuron determinant Col, which has a dynamic expression pattern in the NB5-6T lineage (Baumgardt et al., 2009). In wild type, Col is initially expressed in all four newly-born Ap neurons, and plays a critical role in activating Ap and Eya. This leads to transient specification of a generic Ap neuron fate. Subsequently, there is a critical down-regulation of Col during St17-18hAEL in the Ap2, Ap3 and Ap4/FMRFa neurons, while expression of Col is maintained in Ap1/Nplp1 throughout larval stages. In the Ap1/Nplp1 neurons, Col plays a critical multi-step feedforward role, activating Nplp1 and specifying the Ap1/Nplp1 fate (Baumgardt et al., 2007). Conversely, down-regulation of Col in the Ap2, Ap3 and Ap4 cells allows for the establishment of alternate terminal cell fates (Baumgardt et al., 2009). Analyzing Col expression in *svp* mutants, we found that the initial activation of Col was unaffected, and Col expression was observed in all newly-born Ap neurons (Fig. 5E, 5K, 5M). However, the subsequent down-regulation of Col did not occur in *svp* mutants, and Col was observed in 3-4 cells in *svp* embryos (Fig. 5F, 5L, 5M). Thus, the principal effects in *svp* mutants are a loss of Nab expression, and a failure to down-regulate Col (Fig. 5N).

Combined with the analysis of the expression of the Nplp1 and FMRFa neuropeptides, as well as of the Dimm regulator, these findings are consistent with an expansion of the early Ap window, the Ap1/Nplp1 cell fate, in *svp* mutants. This notion is furthermore supported by the highly restricted expression of Svp: initially being expressed in all four Ap neurons, and gradually restricted to the central Ap window cells, Ap2 and Ap3.

***seven-up* misexpression suppresses cell fates in both the early and late Apterous window**

The *svp* mutant analysis suggests that *svp* acts to suppress Col in the Ap2 and Ap3 neurons at later stages of Ap neuron differentiation, thereby preventing the feedforward action of *col*, which would otherwise result in Ap1/Nplp1 terminal cell fate. In addition, the loss of FMRFa in *svp* may be interpreted as *svp* having a critical role also in specifying the last-born Ap neuron cell fate, Ap4/FMRFa. However, since there is an apparent expansion of the early Ap window in *svp* mutants, the loss of FMRFa may merely reflect a secondary effect. To address these issues further, we misexpressed *svp* using a late postmitotic driver, *ap^{Gal4}*, a driver that commences at St16 i.e., after all four Ap neurons are born and have acquired their early generic cell identity (Allan et al., 2003). We found that misexpression of *svp* from this postmitotic driver had strong effects upon Ap1/Nplp1 neuron terminal differentiation, with a complete loss of Dimm and Nplp1 expression (Fig. 6B, 6D, 6E-F). We also noted a partial loss of Col expression from the Ap1/Nplp1 neuron (Fig. 6A, 6C, 6E). Strikingly however, we also observed a loss of expression of Dimm from the Ap4/FMRFa neuron, as well as loss of the FMRFa neuropeptide (Fig. 6D, 6E-F).

The *svp* gain-of-function phenotypes are in line with the loss-of-function phenotypes with respect to the role of *svp* in repressing the Ap1/Nplp1 cell fate. However, *svp* is also normally down-regulated from the Ap4/FMRFa cells, and misexpression of *svp* led to suppression also of the Ap4/FMRFa fate.

The late expression of Seven-up is controlled by the late temporal genes *castor* and *grainyhead*

It is currently not known how the first pulse of Svp expression is controlled. To address the activation of Svp in the second pulse, we analyzed Svp expression in the pertinent mutant backgrounds. These studies revealed a critical input from both the *cas* and *grh* temporal genes (Fig. 7A-C, 7G). In contrast, mutants for the *sqz* and *nab* sub-temporal genes, as well as the *col* determinant displayed no numerical loss of Svp, although there was reduced intensity of Svp expression (Fig. 7D-H). Thus, the primary upstream regulator of Svp expression in the second pulse within NB5-6T is *cas* and *grh* (Fig. 7I).

DISCUSSION

We find that Svp is expressed in two pulses and plays two different roles in the NB5-6T lineage (Fig. 8). Initially, Svp is expressed briefly in the early part of this lineage, where it acts to control the down-regulation of the first temporal factor, Hb. Subsequently, Svp is expressed in the late part of this lineage, in the Ap window, in a highly dynamic fashion: initiated in all four Ap neurons, to be down-regulated in the first- and last-born Ap cells. In the second expression phase, Svp acts to suppress Col, thereby preventing the first-born Ap neuron fate, Ap1/Nplp1, from being established in the subsequently born Ap2 and Ap3 neurons. Misexpression studies further indicate that Svp also suppresses the last-born Ap neuron fate, Ap4/FMRFa, from being established in Ap2/3.

The early role of Seven-up: a temporal “switching factor”

Previous studies of Svp demonstrated that it is expressed in a brief pulse in the majority of early embryonic neuroblasts, where it acts to suppress Hb, thereby allowing for the switch to the next stage of temporal competence (Kanai et al., 2005; Mettler et al., 2006). Recently, studies have identified additional factors involved in the down-regulation of Hb; the pipsqueak-domain proteins Distal antenna and Distal antenna-related (herein referred to collectively as Dan) (Kohwi et al., 2011). Dan is expressed somewhat earlier than Svp, and is also maintained in a longer pulse. *svp* and *dan* do not regulate each other, and although they can be activated by ectopic *hb* expression, neither Svp nor Dan expression is lost in *hb* mutants. This raises the intriguing questions of how Svp and Dan are activated during early stages of lineage progression, and how they become down-regulated at the appropriate stage.

Another interesting complexity with respect to Svp expression and function pertains to the fact that the Hb window is of different size in different lineages. For example, in NB6-4T and NB7-3, Hb is down-regulated in the neuroblast immediately after the first division (Isshiki et al., 2001; Novotny et al., 2002), while in NB5-6T, Hb expression is evident during three divisions (Baumgardt et al., 2009). In line with this, we do not observe Svp expression in NB5-6T until stage 10, when the neuroblast has already gone through two rounds of division (Fig. 3). How the on- and offset of Svp, and perhaps Dan, expression is matched to the specific lineage progression of each unique neuroblast lineage, to thereby allow for differing Hb window sizes, is an interesting topic for future studies.

The late role of Seven-up: a “sub-temporal” factor

We find that Svp is re-expressed in the NB5-6T lineage in a second pulse. In contrast to the early pulse of Svp expression, where there is no evidence for temporal genes controlling Svp, we do find that the second pulse of Svp expression is regulated by the temporal genes *cas* and *grh*. However, we do not find that *svp* is critical for the expression of Cas or Grh. Instead, we find that *svp* participates in the sub-division of the Cas/Grh temporal window i.e., the Ap window. Based upon that Svp is regulated by temporal genes, and acts to sub-divide a broader temporal window it could be referred to as a “sub-temporal” factor in the latter part of the NB5-6T lineage.

The expression of Svp is dynamic also in the second pulse of expression, commencing in the neuroblast at St14 – after the three first Ap neurons are born – and being maintained in the neuroblast until it exits the cell cycle at St15. At late St14 and 15, Svp expression becomes evident in all four Ap neurons, but it is rapidly down-regulated from Ap1 and Ap4 during stages 16 and 17 (Fig. 3). Svp is however maintained in the Ap2 and Ap3 neurons into late embryogenesis. The role of *svp* in the Ap window appears to be to ensure proper specification of the Ap2/3 interneurons, generated in the middle of the Ap window. This is achieved by *svp* suppressing the first- and last-born Ap neuron fates: the Ap1/Nplp1 and Ap4/FMRFa fates. With regards to the suppression of the Ap1 fate, one critical role for *svp* is to suppress Col expression in Ap2/3. Importantly, the temporal delay in Svp expression when compared to Col – commencing two stages after Col in the Ap neurons – allows for Col to play its critical early role in Ap neuron specification: activating Ap and Eya (Fig. 8). The timely suppression of Col in Ap2/3 is mediated also by *sqz* and *nab* (Baumgardt et al., 2009), and the loss of Nab expression in *svp* mutants may be a contributing factor to the failure of Col down-regulation in *svp*. However, the potent function of *svp* in suppressing Ap1/Nplp1 fate when misexpressed postmitotically from *ap^{Gal4}* does not appear to require Nab, since Nab is not ectopically expressed in these experiments (not shown). Thus, *svp* may act via several routes to prevent Ap1/Nplp1 fate from being established in the Ap2/3 cells: by suppressing Col and by activating Nab.

Regarding the second role of *svp* in the Ap window – the suppression of the Ap4/FMRFa fate – it is less clear what the target(s) may be. However, a common denominator for both the

Ap1/Nplp1 and the Ap4/FMRFa neurons is the expression of Dimm. Dimm, a basic-helix-loop-helix protein, is a critical determinant of the neuropeptidergic cell fate, and also controls high-level neuropeptides expression in many neuropeptide neurons (Hewes et al., 2003; Park et al., 2008). Both *svp* loss- and gain-of-function results in robust effects upon Dimm expression in the NB5-6T lineage, indicating that Dimm is an important target for *svp*. However, *dim* mutants only show reduced levels of FMRFa expression (Hewes et al., 2003), and thus *svp* likely regulates additional targets to prevent the Ap4/FMRFa cell fate in the Ap2/3 neurons.

How global are the roles of *seven-up*?

The early role of *svp*, in its first expression pulse, is to suppress Hb expression. Svp is expressed transiently by most if not all neuroblasts, and the regulation of Hb also appears to be a global event. Similarly, the second pulse of Svp expression has been observed in many lineages, although the role for *svp* in this later pulse was hitherto unknown. Our findings of a role for *svp* as a sub-temporal gene in the latter part of the NB5-6T lineage indicates that *svp* may play such roles in many lineages. However, it should be noted that we do not observe global changes in Col, Dimm and Eya expression in the embryonic CNS (not shown). Thus, unlike the more universal role of *svp* in regulating Hb during the first pulse, the putative sub-temporal function of the second pulse of *svp* expression in other lineages must be highly context-dependent and involving other targets.

In mammals, the *svp* orthologs *COUP-TFI* and *-II* are expressed dynamically in the developing CNS (Qin et al., 2007; Yamaguchi et al., 2004). Functional studies reveal a number of important roles for *COUP-TFI/II* during nervous system development, and mutant mice display aberrant neuro- and gliogenesis, accompanied by axon pathfinding defects (Qiu et al., 1997; Yamaguchi et al., 2004). Intriguingly, recent studies have revealed that *COUP-TFI/II* acts in a temporal manner to control the timing of generation of sub-classes of neurons and glia in the developing mouse brain (Naka et al., 2008; Tomassy et al., 2010). Given that the other genes described in this study are also conserved, it is tempting to speculate that temporal and sub-temporal cascades similar to those outlined here are also utilized in the mammalian CNS during development.

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MATERIAL AND METHODS

Fly Stocks

Fly stocks were maintained at 25°C on standard medium. The following stocks were used: *svp*^{e22} (also called *svp*¹) (Hiromi et al., 1993). *svpe*³⁰⁰ (also called *svp*²) (Mlodzik et al., 1990). *svp*^{11C115} (this work). *UAS-svp1 1.12* (Kramer et al., 1995) containing *svp* type 1 cDNA (Mlodzik et al., 1990). *UAS-svp1.36* (provided by Y. Hiromi). *FMRFa-EGFP* (C.U. and S.T., unpublished). *ladybird early* fragment K driving *lacZ* (referred to as *lbe(K)-lacZ*) (provided by K. Jagla) (De Graeve et al., 2004). *ladybird early* fragment K driving *Gal4* (referred to as *lbe(K)-Gal4*) (Baumgardt et al., 2009). *UAS-nls-myc-EGFP* (referred to as *UAS-nmEGFP*), *UAS-myc-EGFP-farnesylation*, *sqz*^{Df}, *sqz*^{ie}, *UAS-sqz* (Allan and Thor, 2003). *ap*^{md544} (referred to as *ap*^{Gal4}) (O'Keefe et al., 1998). *ap*^{rK568} (referred to as *ap*^{lacZ}) (Cohen et al., 1992). *gsb*⁰¹¹⁵⁵ (referred to as *gsb*^{lacZ}) (Duman-Scheel et al., 1997), a marker for neuroblast lineages in rows 5 and 6 (Buenzow and Holmgren, 1995; Duman-Scheel et al., 1997; Gutjahr et al., 1993; Skeath et al., 1995). *elav-Gal4* (provided by A. DiAntonio) (DiAntonio et al., 2001). *cas*⁴¹ and *cas*⁴³ (Mellerick et al., 1992), and *UAS-cas* (Kambadur et al., 1998), (both provided by W. Odenwald). *grh*^{IM} (Nusslein-Volhard et al., 1984). *Df(2R)Pcl7B* (referred to as *grh*^{Df}). *col*¹, *col*³ (Crozatier and Vincent, 1999) (provided by A. Vincent). *nab*^{SH143}, *nab*^{R52}, *UAS-nab* (provided by F.J. Díaz-Benjumea) (Terriente Felix et al., 2007). Mutants were kept over *CyO*, *Act-GFP*; *CyO*, *Dfd-EYFP*; *TM3*, *Ser*, *Act-GFP*; *CyO*, *twi-Gal4*, *UAS-GFP*; *TM3*, *Sb*, *Ser*, *twi-Gal4*, *UAS-GFP*; or *TM6*, *Sb*, *Tb*,

Dfd-EYFP balancer chromosomes. As wild type, *OregonR (iso2)* was often used. Unless otherwise stated, flies were obtained from the Bloomington Drosophila Stock Center.

Immunohistochemistry

The following antibodies used were: Mouse α -Svp (1:50) (Kanai et al., 2005) (provided by Y. Hiromi). Guinea pig α -Col (1:1,000), guinea pig α -Dimm (1:1,000), chicken α -proNplp1 (1:1,000) and rabbit α -proFMRFa (1:1,000)(Baumgardt et al., 2007). Rat α -Grh (1:1,000) (Baumgardt et al., 2009). Rabbit α -Nab (1:1,000)(Terriente Felix et al., 2007) (provided by F.J. Díaz-Benjumea). Rabbit α -Cas (1:250)(Kambadur et al., 1998) (provided by W. Odenwald). Guinea pig α -Deadpan (1:1,000) (provided by J. Skeath). Rat monoclonal α -Gsbm (1:10)(provided by R. Holmgren). Rabbit α -Hunchback (1:1,000)(provided by R. Pflanz). Rat α -Sqz (1: 750) (Tsuji et al., 2008) (provided by T. Isshiki). Rabbit α - β -Gal (1:5,000; ICN-Cappel, Aurora, OH, US). Mouse α -myc (1:2,000; Upstate/Millipore, Billerica, MA, US). Chicken α - β -Gal (1:1,000; Abcam, Cambridge, UK). Mouse α -Eya 10H6 (1:250)(Developmental Studies Hybridoma Bank, Iowa City, IA, US). All polyclonal sera were pre-absorbed against pools of early embryos. Secondary antibodies were conjugated with AMCA, FITC, Rhodamine-RedX or Cy5, and used at 1:200 (Jackson ImmunoResearch, PA, US). Embryos were dissected in PBS, fixed for 25 minutes in 4% PFA, blocked and processed with antibodies in PBS with 0.2% Triton-X100 and 4% donkey serum. Slides were mounted in Vectashield (Vector, Burlingame, CA, US). For embryonic stages 9-12, embryos were stained as whole-mounts, using the same protocol. Embryos were staged according to (Campos-Ortega, 1997).

Confocal Imaging and Data Acquisition

Zeiss LSM 5 or Zeiss META 510 Confocal microscopes were used to collect data for all fluorescent images; confocal stacks were merged using LSM software or Adobe Photoshop. Where immunolabeling was compared for levels of expression, wild-type and mutant tissue was stained and analyzed on the same slide. Statistical analysis was performed using Microsoft Excel, and bar graphs generated using GraphPad Prism software. Statistical Methods Quantifications of observed phenotypes were performed using Student's two-tailed t test, assuming equal variance.

Figure Legends

Fig. 1.

A genetic screen for FMRF-GFP expression in Apterous neurons identifies *seven-up*.

(A) Previous studies identified several regulatory genes specifically expressed in subsets of Ap neurons, acting to specify their identities (see text for references). (B) Model of the NB 5-6T lineage based on previous studies (Baumgardt et al., 2009). The four Ap cluster neurons are the last-born neurons, and are generated within a Cas/Grh temporal window. (C-D) Expression of *FMRFa-EGFP* in living late embryos, in wild type (C) and *svp* mutants (D). In wild type, expression of EGFP is clearly observed in the six thoracic Ap4/FMRFa neurons. In *svp*, expression of EGFP is completely lost. Genotypes: (C) $w^{1118};;FMRFa-EGFP, UAS-mRFP$. (D) $w^{1118};;FMRFa-EGFP, UAS-mRFP, svp^{11C115}$.

Fig. 2.

Seven-up is critical for down-regulating Hunchback in the VNC and in NB5-6T.

(A-B) Hb expression at St14 in control (A) and *svp* mutant (B). *svp* mutants show a failure to down-regulate Hb in the VNC. (C-E) Staining for Hb in NB5-6T at St14 in control (C) and *svp* mutants (D-E). The NB5-6T is identified as the anterior- and lateral-most neuroblast within the *gsb^{lacZ}* domain (red), as well as by cell size and staining for Deadpan (blue). At St14, Hb expression is not detected in the control embryo. However, in *svp* mutants, Hb is still present in 36% of hemisegments (D), while being absent in 64% (E). Genotypes: (A, C) *OregonR*. (B, D, E) svp^1/svp^{11C115} .

Fig. 3.

Seven-up is expressed in two distinct windows of NB5-6T development.

(A-L) Expression of Svp (red) within NB5-6T, during embryonic development. To the right are side-view graphic representations of the lineage. Images are composed from confocal stacks, in G, H and I subdivided into two sub-stacks, from dorsal (up) to ventral (down). Anterior is up in all images. (A-C) NB5-6T is identified as the anterior- and lateral-most neuroblast within the *gsb^{lacZ}* domain (green), as well as by cell size and staining for Deadpan (white) (A-C). (D-I) Alternatively, NB5-6T is identified by reporter gene expression driven from NB5-6 specific

lbe(K) enhancer. Ap1, Ap2, Ap3 and Ap4 neurons are identified by different levels of Nab or Cas (blue) and Col (not shown) (Baumgardt et al., 2009). (J-L) During later stages, Ap1, Ap2, Ap3 and Ap4 neurons are identified by expression of *ap^{lacZ}* (green), different levels of Cas staining (J; blue), Dimm (K and L; blue) and Col expression (not shown) (Baumgardt et al., 2009). The early pulse of Svp expression commences at St10 (B), but is rapidly lost, and not detectable in the neuroblast until late St14 (G), when the late pulse of Svp expression commences. The second pulse is dynamic; firstly observed in the newly-born Ap4/FMRFa neuron and at St15 in all four Ap neurons (I). From St16, Svp is no longer evident in Ap1 (J), and at St17 is also lost from Ap4 (K). However, Ap2 and Ap3 express Svp until the end of the embryogenesis (L). (M) Cartoon depicting the expression of Svp in the NB5-6T lineage. Genotypes: (A-C) *gsb^{lacZ}/+*. (D-I) *lbe(K)-Gal4, lbe(K)-Gal4/UAS-nmEGFP*. (J-L) *ap^{lacZ}/+*.

Fig. 4.

***seven-up* is critical for Apterous neuron specification.**

Expression of the Ap cluster determinants Eya and Dimm, and of the terminal identity markers FMRFa and Nplp1, in control and *svp* mutants: stage 18hAEL embryonic VNCs (anterior up; brackets outlining the three thoracic segments). The observed phenotypes are summarized in the cartoon (O) and in a graphical representation of the quantified results (P): $n \geq 10$ VNC in all genotypes: asterisks (*) denote significant difference compared to control (Student t-test, $p < 0.001$). Expression of Eya reveals that the Ap cluster is not generated in some thoracic hemisegments in *svp* mutants. However, in the cases in which the cluster is born, it displays an increase in the number of cells with respect to the control (E, L-N, O and P). Staining against FMRFa shows a complete loss of expression in *svp* mutants (F, L, O and P), whereas ectopic Nplp1 expressing cells in the Ap clusters are revealed by proNplp1 staining (G, M, O and P). Expression of Dimm reveals ectopic Dimm-positive neurons within the Ap cluster (N, O and P). Genotypes: (A-D, I-K) *OregonR*, (E-H, L-N) *svp¹/svp^{11C115}*.

Fig. 5.

***seven-up* both positively and negatively controls several Apterous neuron determinants.**

(A-L) Expression of Ap neuron determinants Cas, Grh, Sqz, Nab and Col, in control and *svp* mutant thoracic hemisegments, at St16 (A-E, G-K) or 18hAEL (F, L). Eya was used to visualize

Ap neurons. (M) Quantification of the observed phenotypes ($n \geq 10$ hemisegments; asterisks (*) denotes significant difference compared to control (Student t-test, $p < 0.05$). (N) Cartoon summarizing the phenotypes of the quantified results. Cas is expressed in three out the four Ap neurons in control (A), and its expression appears somewhat reduced in *svp* (G, M and N). Expression of Grh is limited to one Ap neuron in both control (B) and *svp* mutant embryos, although this cell expresses weakly in the mutant Ap neurons (H). Sqz is detectable in three of the Ap neurons in control (C) and is not lost in *svp* mutants. Nab is expressed by three Ap cells in the control (D). However, Nab staining is not detectable in *svp* mutants (J). Col expression is detected in all neurons of the Ap cluster in control at stage 16 (E), but it is absent from some cells in *svp* mutants (K, M and N). At stage 18h AEL, Col expression is restricted to one cell in the Ap cluster in the control (F), whereas it is expressed in additional cells in *svp* mutants (L, M and N). Genotypes: (A-F) *OregonR*. (G-L) *svp¹/svp^{11C115}*.

Fig. 6.

Misexpression of *seven-up* blocks the Ap1/Nplp1 and Ap4/FMRFa cell fates.

(A-D) Expression of Eya and Dimm in the Ap cluster at 18hAEL, in control and *svp* misexpression from the post-mitotic driver *ap^{Gal4}*. (A) In control, one of the four Ap cluster cells (Ap1/Nplp1) expresses Col. (C) Post-mitotic expression of *svp* reduces Col expression. (B) In control, the Ap1 and Ap4 neurons express Dimm. (D) In *svp* misexpression, Dimm is completely lost from both neurons. (E-F) Quantification of the observed phenotypes ($n \geq 30$ hemisegments in all genotypes; asterisks (*) denote significant difference compared to control (Student t-test, $p < 0.001$). Genotypes: (A-B) *ap^{Gal4}/+*. (C-D) *ap^{Gal4}/UAS-svp*.

Fig. 7.

The temporal genes *castor* and *grainyhead* control *Seven-up* expression during late NB5-6T lineage development.

(A-F) Svp expression in mutants for Ap neuron determinants. St16 embryonic VNCs; anterior up. (A) At St16, Svp is expressed by Ap2, Ap3 and Ap4. The expression in Ap4 is stronger than in Ap2 and Ap3, and is not detected at all in Ap1. Svp is completely lost in *cas* mutants (B), is significantly reduced in *grh* mutants (C), but unaffected in *sqz* mutants (D). (G-H) In *nab* and *col* mutants, Svp is expressed in the proper cells, but weaker than in control. (G) Quantification of

Svp expression ($n \geq 10$ VNC in all genotypes). Asterisks (*) denote significant difference compared to control (Student t-test, $p < 0.001$). (H) Quantification of staining intensity of Svp in control, *nab* and *col* mutants ($n \geq 10$ VNCs). The asterisk (*) denotes that staining intensity of Svp is significantly affected both in *nab* and *col* mutants (Student t-test, $p \leq 0.001$). Wild type and mutant VNCs were stained and analyzed on the same slide. (I) Cartoon summarizing our findings. See text for details. Genotypes: (A) *OregonR*. (B) *lbe(K)-lacZ; cas^{Δ1}/cas^{Δ3}*. (C) *grh^{IM}/grh^{Df}, lbe(K)-Gal4/UAS-nmEGFP*. (D) *lbe(K)-Gal4/UAS-nmEGFP; sqz^{ie}/sqz^{Df2411}*. (E) *lbe(K)-Gal4/UAS-nmEGFP; nab^{SH143}/nab^{R52}*. (F) *col¹/col³*.

Fig. 8.

Model of NB5-6 lineage development and the role of *seven-up*.

The NB5-6T lineage generates an early lineage of neurons and glia (middle), and undergoes the typical temporal progression during these stages. Here, Svp acts to ensure the proper down-regulation of Hb (left). As the lineage progresses into the Cas/Grh window, generation of the four Ap neurons commences, with the precise orchestra of regulatory gene expression playing out in the neuroblast and in the developing Ap neurons (top). Svp (red) is re-expressed during these stages, and shows dynamic expression, being turned on in the neuroblast first, in all four Ap neurons, and down-regulated and maintained only in the Ap2 and Ap3 neurons. During this second phase of Svp expression, our findings indicate that *svp* acts to ensure that the Ap2/3 cell fate is established in the middle Ap window, by suppressing Col and Dimm.

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Figure 1

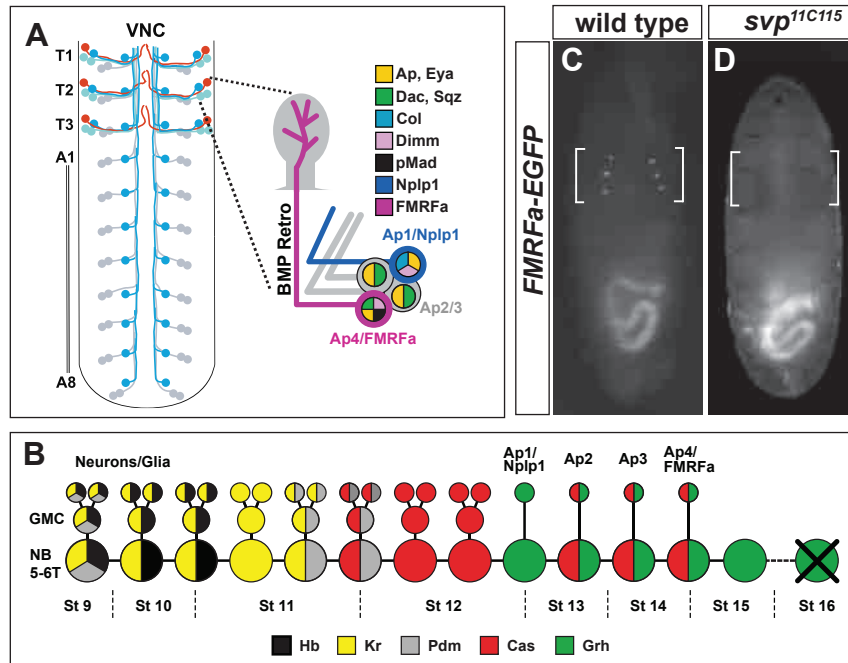


Figure 2

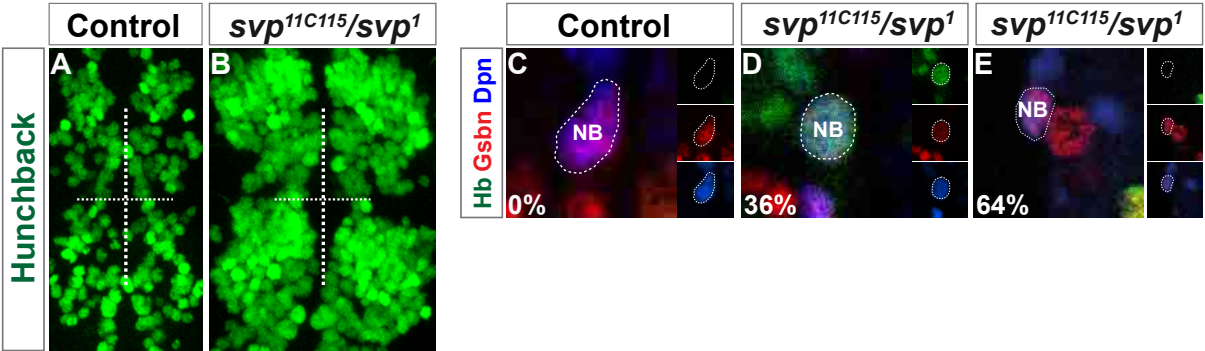


Figure 3

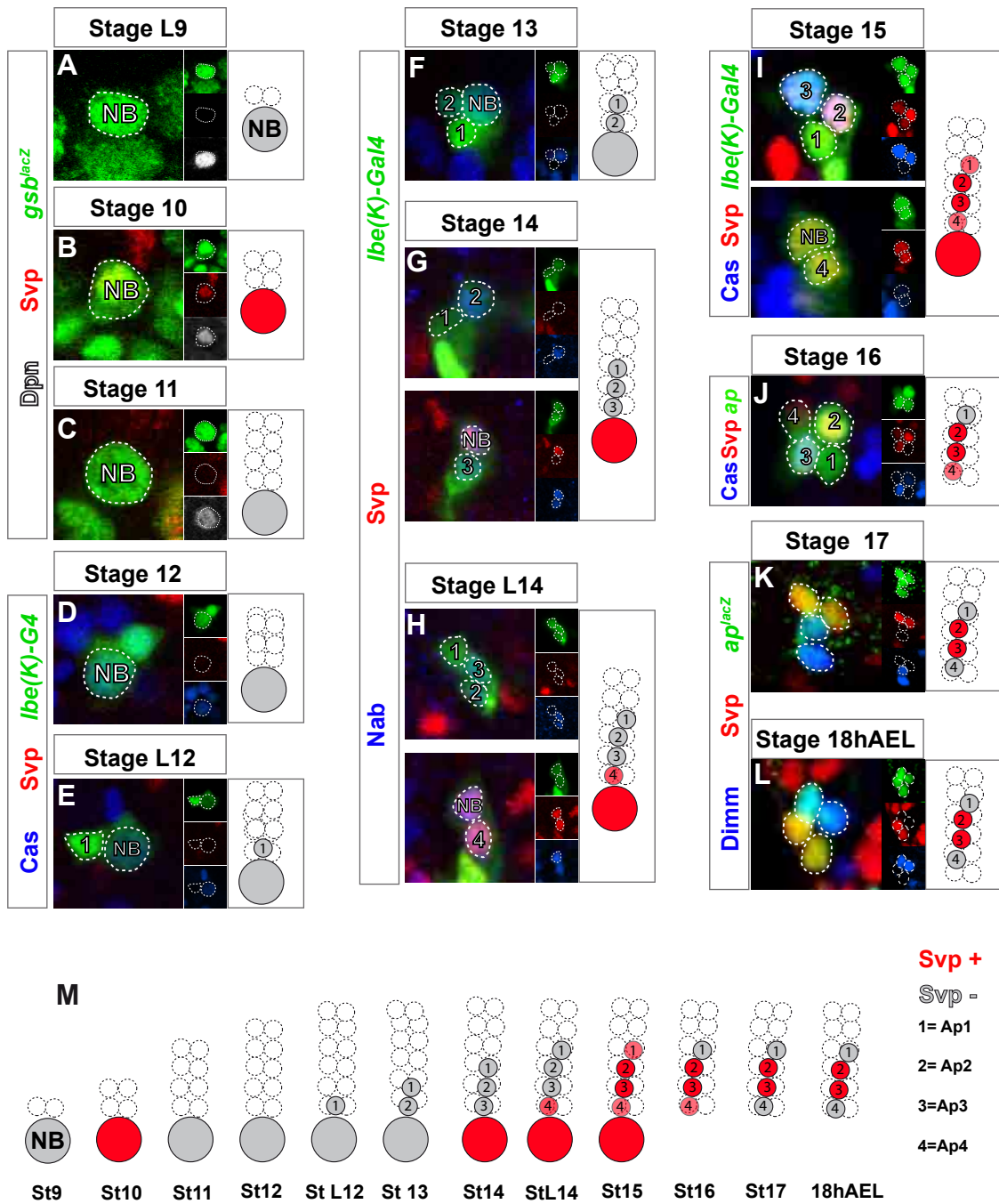


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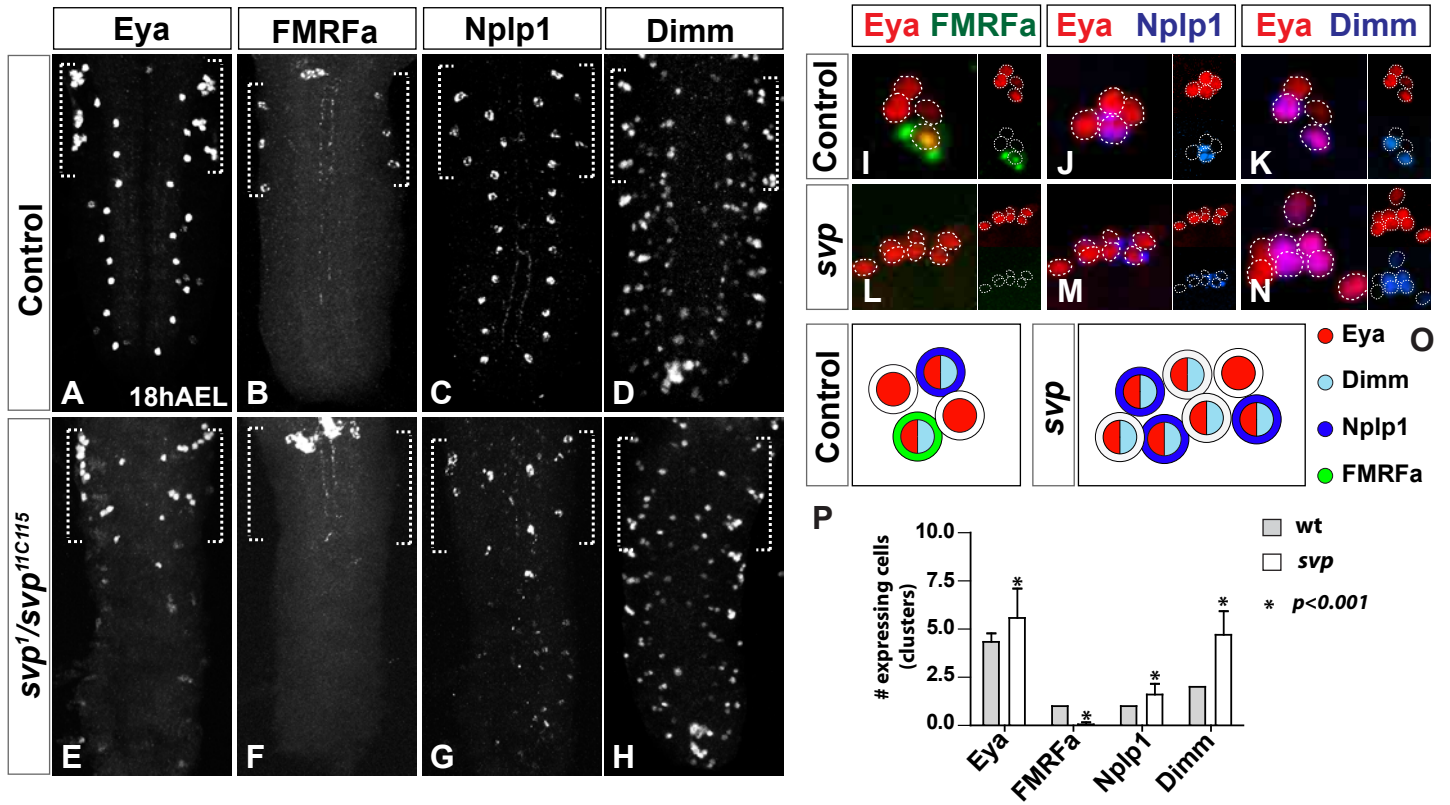


Figure 5

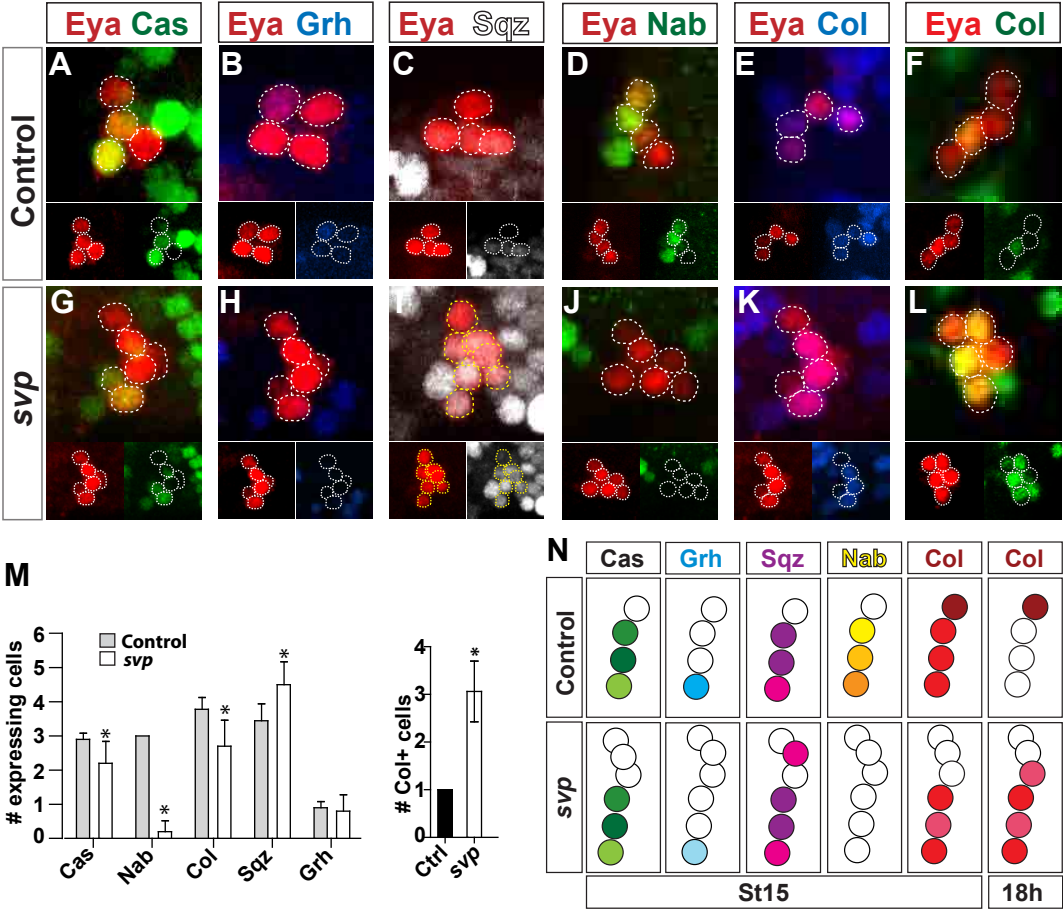


Figure 6

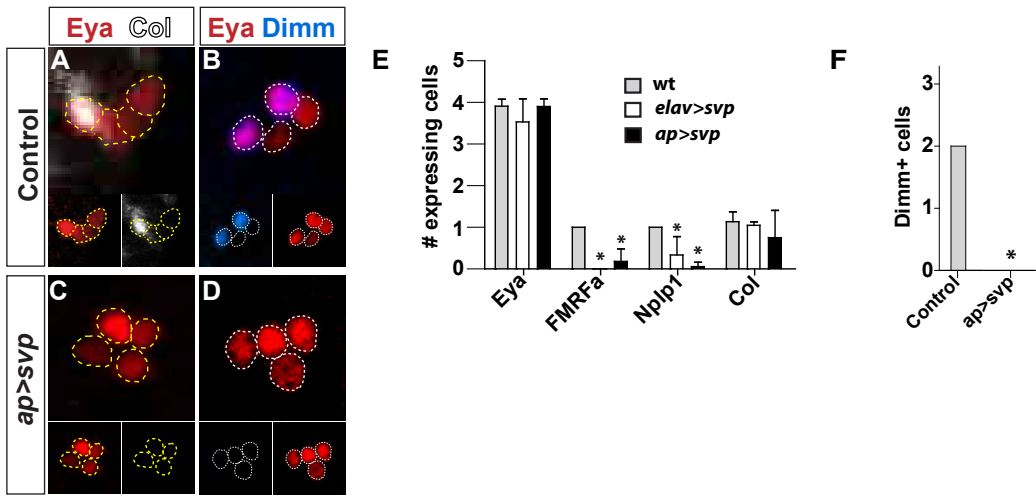


Figure 7

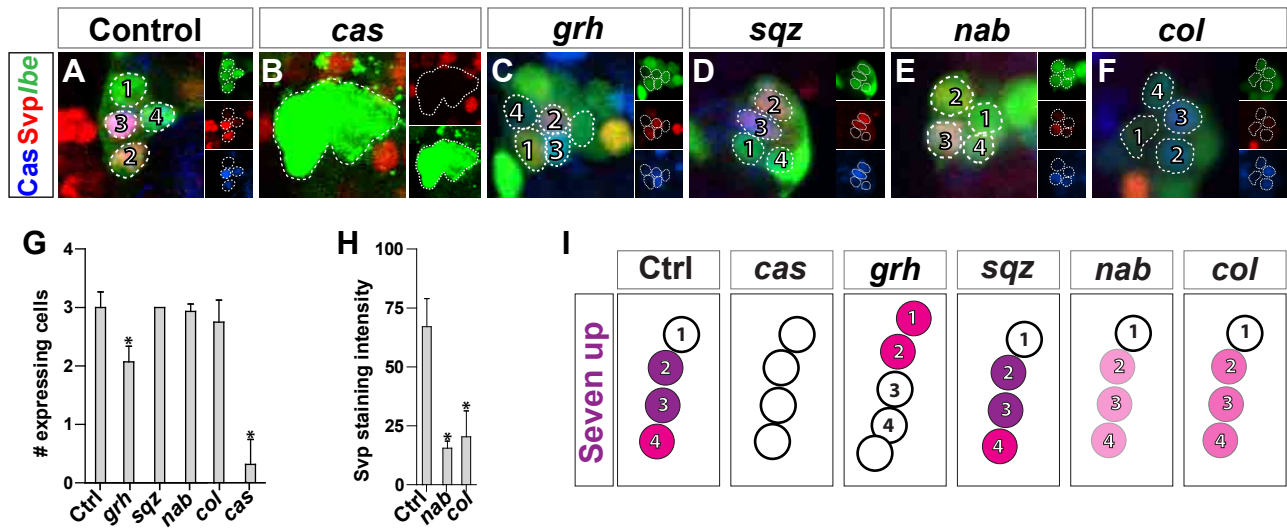


Figure 8

